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PRINCIPAL INVESTIGATOR: David J. Shapiro, Ph.D.

CONTRACTING ORGANIZATION: University of Illinois
Champaign, Illinois 61820

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13. ABSTRACT *(Maximum 200 words)*

The objective of this project is to develop and use novel ER mutants to test a two-part hypothesis. First, that the estrogen-independent growth of breast cancer cells involves the estrogen-independent expression of genes which are normally estrogen regulated. Second, that suppressing the expression of these genes will block the growth of breast cancer cells. To test these ideas, we set out to develop a novel type of estrogen receptor chimera which will efficiently and quantitatively suppress both estrogen-dependent and estrogen-independent expression of estrogen-regulated growth stimulatory genes. We used information from a genetic selection done using our modified P222 challenge phage system to identify mutations which lead to steroid receptor DNA binding domains with a strongly enhanced affinity for the estrogen response element (ERE). To repress transcription of estrogen receptor regulated genes we created chimeras in which full length ER or the ER DNA binding domain is fused to a powerful KRAB repressor domain. We showed that these chimeras form powerful, easily-regulated, ligand-dependent repressors. Wild-type ER, fused to KRAB domains, was unable to repress transcription of the native pS2 gene, while a KRAB-ER chimera containing a set of up-binding mutations we identified in our challenge phage selections was a powerful ligand-dependent repressor of both basal and estrogen-induced transcription of the pS2 gene.

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David J. Shapiro 8/29/99

PI - Signature Date

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INTRODUCTION

Estrogens, acting through the estrogen receptor (ER), play a critical role in regulating the growth and metastases of breast cancers. Growth promotion by estrogen is thought to involve direct estrogen receptor-mediated regulation of the expression of several genes important in cell growth, including those encoding some growth factors (such as TGF-α, IGF1, and their receptors), some early response genes (such as c-myc, and cyclin D1), and other genes, including the progesterone receptor gene. Interference with estrogen activity, usually based on antiestrogens, such as tamoxifen, or on aromatase inhibitors, therefore represents a mainstay in breast cancer treatment. While antiestrogen therapy is often effective initially, the tumors almost always eventually progress to estrogen-independent growth. This limits the long-term utility of endocrine therapies. It is often accepted that the ability of the 17β-estradiol-ER complex to influence the growth and metastases of breast cancer cells is due to its ability to regulate the expression of specific genes. the estradiol-ER complex acts directly to induce the expression of a set of "early growth response genes" including c-myc, cyclin D1 and TGF-α, and other genes important in cell growth, by directly interacting with these genes. These early genes and the products of other directly regulated genes, may also initiate a regulators cascade leading to the regulation of downstream genes important in growth control in breast cancer cells. It has been widely proposed that the 17β-estradiol-ER complex induces breast cancer cell growth by directly or indirectly regulating the expression of genes important in cell growth control. **If genes critical to growth control in breast cancer cells are directly induced by the estradiol-ER complex, then repression of estrogen-dependent and estrogen-independent transcription of these genes should block estrogen stimulated growth of the breast cancer cells.** Although it is known that estrogen growth autonomous cells synthesize high levels of growth factors that are normally under estrogen regulation, the hypothesis that the high level expression of growth factor genes is responsible for growth of these cells has not been tested directly. We will test this hypothesis by repressing the transcription of these growth factor genes and determining the effect on breast cancer cell growth. If this hypothesis is correct, suppression of the expression of these genes should result in the loss of estrogen-independent growth of these cells, and possibly in their death. In addition, identification of genes as critical for estrogen-dependent or estrogen-independent tumor cell growth provides a basis for the identification of additional agents to suppress their activity.

PROGRESS REPORT

Background

There are two key features relevant to the development of the repressor proteins.

The P22 challenge phage system.

In the bacteriophage P22, the decision between lysis of the infected cells and lysogeny, which allows outgrowth of bacterial colonies is exclusively based on the *Ant* protein, whose production results in lysis of the bacteria. In our initial application, we generated a recombinant phage we generated a recombinant phage with an ERE close to the *Ant* promoter. If a steroid receptor mutant with sufficient affinity binds to this ERE, it will block transcription of the *Ant* gene, producing bacterial colonies. Although conceptually simple, the challenge phage system is technically quite complex, and it required a major development effort in our laboratory (which began in 1991), to modify it so that it could be used successfully with vertebrate proteins. (Our initial paper describing up-binding mutants with altered specificity and enhanced affinity for the ERE produced using this system has just appeared: Chusacultanachai, S. et al., 1999. *J. Biol. Chem.* **274**, 23591-23598.)

Information gained from these mutants was critical to the production of the repressor proteins.

B2. The KRAB repressor

When tethered to a DNA binding domain THE KRAB repressor domain, a ~70 amino acid domain found in a substantial number of DNA binding proteins can efficiently suppress transcription of genes containing strong binding site for the protein.

RESEARCH PROGRESS

The Progress Report contains as the second Appendix the complete text and figures of a manuscript which we submitted for publication describing the properties of our ER-KRAB repressor chimeras. This paper is now undergoing revision. A key outcome of this work was our finding that a KRAB-ER-KRAB chimera, which efficiently repressed transcription of synthetic genes containing multiple EREs, was unable to repress expression of the native pS2 gene, which contains a single imperfect ERE. In contrast, a chimera into which we inserted a set of mutations we identified in our challenge phage selection as conferring enhanced binding to the ERE, was a potent repressor of both basal and estrogen-dependent expression of the pS2 gene. Although pS2 provides a useful prognostic indicator in breast cancers, repression of pS2 expression is not expected to block the growth of breast cancer cells

In addition, we describe below an approach to employing hybrid molecules targeted to two different half sites. We used two up-binding mutants covalently joined with a flexible amino acid linker, and showed that this dimeric protein is a highly potent gene repressor.

Dimerizing a Genetically Selected Up-binding mutant-KRAB Chimera with a Flexible Linker results in a Potent Repressor.

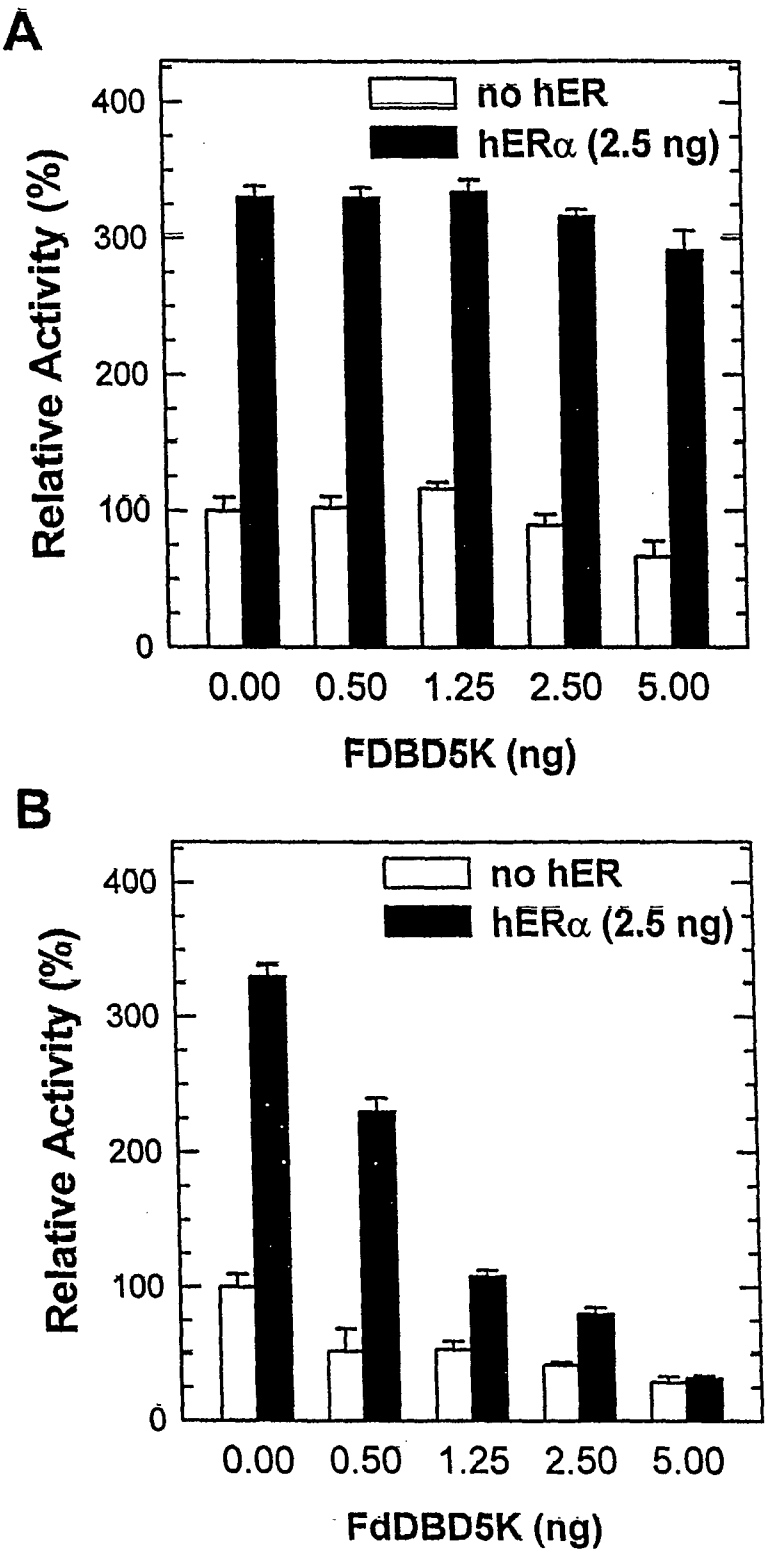
To create an effective transcription repressor , we used a flexible linker to artificially dimerize two copies of the genetically selected specificity switch, ERE up-binding mutant, DBD5, and inserted a copy of the powerful KRAB repressor domain at its c-terminus. Repression was determined by the ability to suppress expression of a constitutively active promoter containing EREs. The linker-dimerized DBD5-KRAB (dDBD5K) chimera is a much more potent repressor of transcription of an ERE-containing gene than the DBD5-KRAB (DBD5K) monomer. DBD5K did not repress expression of the native pS2 promoter, while dDBD5K elicited a dose-dependent repression of both constitutive and estrogen-receptor induced expression of the pS2 test promoter. The linker dimerized protein dDBD5K is specific for genes containing the ERE, and does not repress transcription of a test gene containing the glucocorticoid/progesterone response element (GRE/PRE).
Artificially dimerizing the genetically selected DBDs using a flexible linker results in a dramatic increase in their potency as sequence-specific transcription repressors. In addition, artificially dimerizing the genetically selected mutants allows for the covalent joining of two different proteins, each targeted to a specific DNA sequence. This will greatly increase the specificity and potency of the targeted mutants.

We have begun selections aimed at targeting the TGF-α, c-myc and bcl-2 genes to examine their importance in the growth and apoptosis of breast cancer cells. This year we expect to begin delivering the repressors to breast cancer cells and examining effects on their growth and death.

Figure Legends

Figure 1 dDBD5K suppresses transcription from the natural pS2 promoter. The ability of DBD5K (panel A) and dDBD5K (panel B) to suppress basal (open bars) or estradiol-ER activated transcription (dark bars) of the natural pS2 promoter was examined in human tumor cells transfected with the indicated amount of expression plasmid (0.5, 1.25, 2.5 and 5 ng). The basal activity of the pS2 promoter was set equal to 100%. 17β-estradiol was present in the culture medium at 10 nM.

Figure 2. dDBD5K is unable to repress the Mouse Mammary Tumor Virus (MMTV) promoter. The indicated amounts of dDBD5K expression plasmid (0.5, 1.25, 2.5 and 5 ng) were transfected into human cancer cells along with glucocorticoid and progesterone inducible MMTV-CAT reporter plasmid and in some cases, 2.5 ng of a glucocorticoid receptor expression plasmid (SV40GR). The ability of dDBD5-KRAB to suppress basal (open bars) or GR activated transcription (dark bars) were compared to the MMTV basal activity which was set equal to 100%. 10 nM hydrocortisone was added to the culture medium.


A
no hER
hERα (2.5 ng)
Relative Activity (%)
400
300
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0
0.00
0.50
1.25
2.50
5.00
FDBD5K (ng)
B
no hER
hERα (2.5 ng)
Relative Activity (%)
400
300
200
100
0
0.00
0.50
1.25
2.50
5.00
FdDBD5K (ng)

Fig. 1

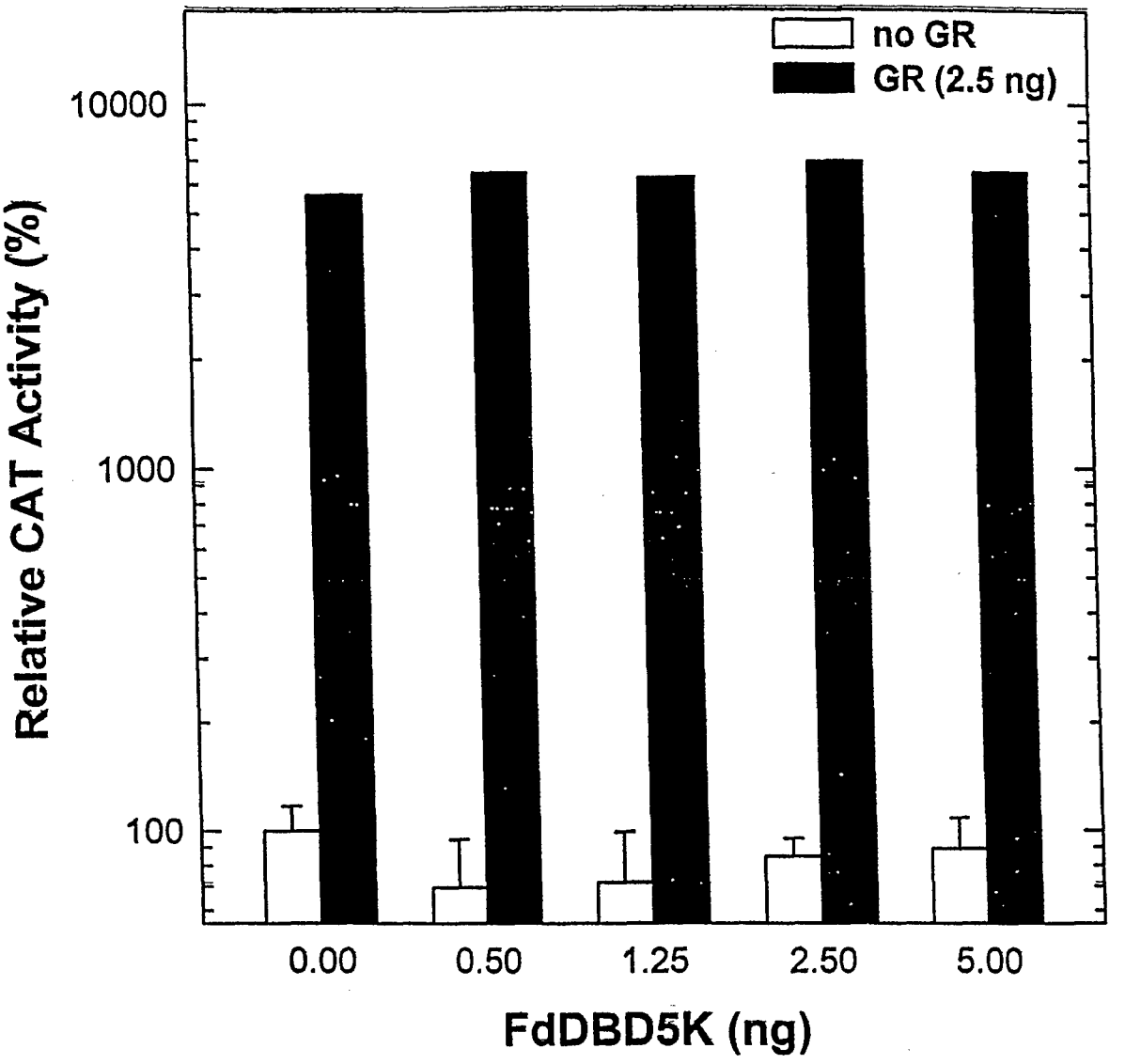
no GR
GR (2.5 ng)
10000
1000
100
Relative CAT Activity (%)
0.00
0.50
1.25
2.50
5.00
FdDBD5K (ng)

Fig. 2

Major Accomplishments.

- We developed and characterized a novel set of ligand-dependent chimeric proteins which repress the expression of genes containing estrogen response elements.

- We demonstrated repression basal and estrogen induced expression of the native pS2 gene, a prognostic indicator in breast cancer.

- Insertion into the chimeras of mutations we identified by our genetic selection as enhancing binding to the estrogen response element was critical was critical for achieving repression of the pS2 gene.

- We developed and demonstrated greatly enhanced repression by chimeras in which two monomers are covalently joined by a flexible linker.

Most Important Problem

- The effort required to select, characterize and introduce into breast cancer cells repressors to several different genes requires multiple researchers and is well beyond the quite limited resources provided for this project.

PROGRESS ON STATEMENT OF WORK

Task 1: Months 1-12: We will prepare potent repressors of estrogen regulated genes (ERG-repressors).

Progress: Potent repressors have been prepared and characterized and manuscript based on that work has been submitted.

Task 2: Months 6-18: We will characterize the ability of the ERG-repressors to suppress transcription of synthetic reporter genes and of endogenous ER-regulated cellular genes.

Progress: The repressors have been characterized and much of the characterization is included in the submitted paper.

Task 3: Months 16-30: We will construct stable cell lines expressing the ERG-repressor.

Progress: Efforts to introduce the repressors into breast cancer cells are under way. In another project, we recently described a novel way to prepare stable cell lines expressing toxic proteins (Zhang, C.C. *et al.*, 1999. *Mol. Endocrinol.*, **13**, 632-643). This approach, which is based on the production of a bicistronic mRNA facilitates these studies.

Task 4: Months 18-36: We will test the ability of the ERG-repressor to suppress the growth of ER^+ MCF-7 cell lines (parental, and antiestrogen resistant, and estrogen-autonomous) and of ER^- 231 cells.

Progress: Studies in this area are just beginning.

Task 5: Months 24-36: We will evaluate the ability of the ERG-repressor to block the growth of solid tumors derived from antiestrogen resistant and estrogen autonomous MCF-7 cells and from 231 cells.

Progress: These studies were not slated to be carried out until the coming year.

Estrogen Receptor-KRAB Chimeras are Potent Ligand Dependent Repressors of Estrogen Regulated Gene Expression.

Georgius de Haan[1§], Sudsanguan Chusacultanachai[1‡], Benita S. Katzenellenbogen[2], and David J. Shapiro[1].

Running title: Estrogen Receptor-KRAB Chimeras are potent transcription repressors

From the [1]Department of Biochemistry and [2]Department of Molecular and Integrative Physiology, University of Illinois at Urbana-Champaign, Urbana IL 61801.

§ Present Address: Women's Health Research Institute, Wyeth Ayerst Research, Radnor, PA.

‡ Present Address: National Center for Genetic Engineering and Biotechnology, 539/2 Gypsum Metropolitan Tower, Bangkok,10400 Thailand

Email djshapir@uiuc.edu

Fax (217)-244-5858

reprint requests to David J. Shapiro

ABSTRACT

To facilitate functional studies of estrogen receptor (ER)-regulated genes, we constructed human ER α (hERα) chimeras which repress transcription of estrogen response element (ERE)-containing promoters. The KRAB (Krüppel associated box) repressor domain was fused to the N and/or C-terminus of wild-type hERα and to hERα mutants defective in AF1, AF2, or both activation functions. All of the hERα-KRAB chimeras were effective transcriptional repressors, indicating that repression by the KRAB domain was dominant over transactivation mediated by AF1 and AF2. Repression was specific for ERE-containing genes. While an ER ligand was required for repression, both estrogens and antiestrogens were effective. The KRAB-hERα-KRAB (KERK) chimera repressed transcription in all cell lines tested on SV40, TK, EF1-α and vitellogenin promoters containing 2 or 4 inserted EREs. A single consensus ERE was sufficient for repression, but the KERK chimera was unable to suppress transcription from the imperfect ERE in the native pS2 promoter. Mutations previously identified in a genetic screen for DNA binding domains exhibiting enhanced binding to the ERE strongly potentiated repression by KERK. The resulting mutant KERK-3M chimera repressed essentially all ER induced transcription and most basal transcription on the pS2 promoter. Therefore, hERα-KRAB chimeras provide a powerful new tool for ligand-dependent regulation of basal and estrogen-dependent transcription of genes containing EREs.

INTRODUCTION

The sex hormone estrogen exerts a wide variety of biological effects, including effects on function and development of male and female reproductive tissues, bone remodeling and the cardiovascular system. In addition to these beneficial effects however, estrogen has also been implicated in breast and uterine cancer. Therefore considerable efforts have gone into understanding the mechanisms by which estrogen regulated genes mediate both these beneficial and detrimental effects, especially since they may provide novel therapeutic targets.

Estrogen exerts it biological effects in different tissues through an interaction with estrogen receptor (ER), a member of the steroid receptor superfamily. Steroid receptors act as ligand dependent transcription regulators and display a modular domain structure in which discrete regions in the receptor are responsible for different functions (1). The most N-terminal part of the receptor is comprised of the A/B domain, which contains a ligand independent transcription activation function (AF1). Region C, the DNA binding domain (DBD), contains two Cis_4 zinc fingers responsible for DNA binding. The hinge region D, provides a flexible loop connecting the DBD to the ligand binding domain (LBD), which comprises the C-terminal end of the receptor. In addition to binding hormone this domain is responsible for receptor dimerization and contains a ligand dependent transcription activation function, AF2. Binding of hormone activates the receptor, which allows the receptor to dimerize, bind the ERE and function as a transcription regulator.

If expression of estrogen regulated genes could be abolished effectively, both the discovery and the elucidation of their role in various physiological processes would be

greatly facilitated. Antiestrogens (For review see: (2) and ER mutants displaying a dominant negative phenotype (3) have been used to suppress ER induced transcription. However, antiestrogens can display significant agonist activity in specific tissue or cell backgrounds (4,5). Recently, a number of hERα mutants displaying a dominant negative phenotype have been described (3,6). These are receptors that are transcriptionally inert and capable of disrupting the transcription activation properties of estrogen receptor when co-expressed. While these hERα mutants and antiestrogens disrupt estrogen induced transcription, they do not affect basal transcription of estrogen regulated genes. We therefore designed novel hERα variants for ligand-dependent repression of the transcription of estrogen response element (ERE)-containing genes.

The repressor module used in this study was the KRAB (Krüppel-associated box) transcription repression domain (7,8) of the Kox1 protein (also named ZNF10; 8,9). The KRAB domain is a highly conserved 75 amino acid region found in approximately one-third of the vertebrate Krüppel-like (Cis_2-His_2) zinc finger proteins (7). The KRAB domain can be subdivided into A and B boxes based on common exon-intron boundaries. The KRAB A box is necessary and sufficient for transcription repression which is potentiated by the KRAB B box (10). The recent discovery that the KRAB domain containing zinc finger protein Kid-1 can disrupt the nucleolus (11) suggests that KRAB can repress RNA polymerase I mediated transcription in addition to the earlier observed repression of RNA polymerase II and III mediated transcription (12,13). While most KRAB containing proteins appear to bind DNA or heteroduplex DNA (14), others appear to be involved in RNA metabolism by either binding RNA directly (15) or via interacting with the hnRP K protein (16).

While the mechanism of transcriptional repression by the KRAB domain is not fully understood, KRAB has been shown to interact with the human corepressors TIF1α, TIF1β (also isolated as KAP-1) and its murine homologue KRIP-1 (17-19). These proteins contain RBCC (RING finger-B boxes-coiled coil), PHD-finger and bromodomain interaction domains. Since these domains are also found in repression complexes implicated in chromatin-mediated transcription repression, it has been suggested that KRAB may act via a similar mechanism (18,20). Interestingly, TIF1α, (21), but not TIF1β, is thought to act as a coactivator of steroid receptor mediated transcription activation by interacting with the AF2 region of ligand-occupied steroid receptors. Since TIF1α uses different domains for steroid receptor and KRAB interaction (20), it may serve a dual role as both a coactivator and a corepressor. Recently TIF1α has been shown to undergo hyperphosphorylation upon interaction with liganded nuclear receptors and then to act as kinase, phosphorylating transcription factors $TF_{II}E\alpha$, $TAF_{II}28$, and $TAF_{II}55$ *in vitro* (22). In addition to repression based on chromatin remodeling, a direct role for the KRAB domain in suppression of transcription has been suggested by the demonstration that KRAB interacts with components of the TATA box-dependent basal transcription machinery (12) and the hyperphosphorylated form of RNA polymerase II (23).

Whatever the precise mechanisms may be by which KRAB functions, it is clear that when tethered to DNA, KRAB suppresses transcription activation mediated by a variety of transcription factors (8,10,12,13,17,24). KRAB functions as a transcription repressor even when its binding site is up to 3 kb from the transcription initiation site (12,13,17,25).

In this work we evaluated the ability of the KRAB domain linked to functional ER containing or lacking its endogenous AF1 and AF2 activation domains to suppress transcription of synthetic genes containing 1-4 copies of the ERE and to suppress transcription through binding to the imperfect ERE in the natural pS2 promoter (26). While ligand modulated repression on synthetic promoters containing multiple binding sites has been demonstrated previously (25,27), ours is the first study to evaluate repression from a single non consensus binding site reflecting natural promoters. We describe ER-KRAB chimeras which exhibit ligand-dependent repression from reporter constructs containing the consensus ERE in several cell and promoter contexts. To suppress transcription from a single imperfect ERE, a novel repressor was developed containing three DBD mutations which we had previously identified in a genetic screen for mutants exhibiting enhanced binding to the ERE (28). This novel ER-KRAB chimera (KERK-3M) is a potent repressor of both basal and estrogen-induced activity of ERE-containing genes.

RESULTS

hER-KRAB Mediated Repression Requires Ligand, EREs and a Functional KRAB Domain

The ability of the KRAB-hERa-KRAB construct (KERK, see also Fig. 2A) to repress transcription of a reporter gene containing the SV40 promoter and enhancer and 4 consensus EREs was tested. This $(ERE)_4$-pGL3-SV40PE reporter plasmid exhibits substantial intrinsic activity referred to as basal transcription, which is further enhanced by ligand activated ER. To establish the effect of ligand on the ability of a KRAB construct to repress transcription, transient transfections were carried out in ER-negative HepG2, human hepatoma cells. Transfections were carried out in the presence or absence of the estrogen, moxestrol, which livers cells metabolize more slowly than E_2. Basal promoter activity of the $(ERE)_4$-pGL3-SV40PE reporter plasmid in the absence of estrogen receptor was used to normalize the data, and was set at 100%. Cotransfected hERa expression plasmid elicited a moxestrol-dependent 3-4 fold induction of luciferase activity (Fig. 1A). While increasing amounts of unliganded ER did not affect transcription, at 20 ng of transfected expression plasmid, KERK elicited a modest 1.6 fold repression of transcription. Full repression (4.8 fold), required the presence of ligand (Fig. 1A). Since KRAB repression was largely ligand-dependent, subsequent studies were carried out in the presence of ligand.

The sequence specificity of repression was shown by the inability of the hERa-KRAB chimera (ERK) to repress transcription from the five GAL4 binding sites in the G_5-pGL3-SV40PE reporter (Fig 1B), and by the inability of GAL4-KRAB to repress transcription from the 4 EREs in the $(ERE)_4$-pGL3-SV40PE reporter (Fig 1C). The reporters were functional, since GAL4-KRAB repressed transcription by 94% from the G_5-pGL3-SV40PE reporter, while hER activated basal transcription 3.8 fold and ERK repressed transcription by 4.5 fold on the $(ERE)_4$-pGL3-SV40PE reporter. The issue of DNA binding specificity was also

addressed by introducing mutations into the DNA recognition helix of the hER-DBD, which shift the specificity from the ERE to the GRE, and thereby prevent ERE binding (6,29). This chimera (ERKmutDBD) no longer repressed transcription on either of the reporter plasmids.

Introducing the mutations E26A, E27A and E28A into the KRAB domain (8) of ERK (ERKmutKRAB) abolished repression (Fig. 1C). The failure of ERKmutKRAB to activate transcription may be due to the inability of these mutations to fully abolish KRAB repression, or to interference with AF2 action by the nearby KRAB domain.

Influence of AF1 and AF2 Mutations on KRAB Repression

The KRAB corepressor protein, TIF1a (17), also interacts with the AF2 region of ligand-occupied hERa (21), and then functions as a coactivator (21). These interactions take place via two distinct interaction domains found within the TIF1a protein, and may interfere with the ability of the KRAB domain to function as a repressor in the presence of AF2. It was therefore of interest to examine whether presenting the KRAB domain in different ways in the context of estrogen receptor chimeras would favor a functional interaction of KRAB and its corepressors, thereby enabling the KRAB domain to operate more effectively as a transcription repressor. To analyze the effect of position and the influence of the ER activation domains on KRAB repression, the KRAB domain was fused in frame at either the N- or the C-terminus and at both ends of hERa (Fig. 2A). To prevent interaction with steroid receptor coactivators, we also employed a number of hERa mutants in which AF1 and/or AF2 activity was ablated. Since the ligand independent activation function AF1 is spread through much of the A/B domain of hERa (30,31), AF1 ablation was achieved by deleting the entire A/B domain (amino acids 1-178, indicated as ΔA/B). Removal of the ligand dependent activation function, AF2, was achieved through introduction of either of two point mutations in the LBD, L540Q and S554fs (Q and FS, respectively). These mutations confer

a dominant negative phenotype on hERa (3), which might further potentiate transcription repression by the KRAB domain.

The ability of the ER-KRAB chimeras to repress transcription was determined by cotransfecting the $(ERE)_4$-pGL3-SV40PE reporter plasmid and increasing amounts (5, 20 or 40 ng) of the expression plasmid encoding each KRAB chimera into HepG2 cells in the presence of 10 nM moxestrol (Fig 2B). Even at the lowest amount transfected, all of the chimeras achieved at least 45% repression and most achieved >55% repression. The differences in repression among the various constructs were modest. All of the ER-KRAB chimeras are therefore effective transcription repressors. Surprisingly, ablation of AF1 and/or AF2 activity had little or no effect on the extent of KRAB repression. For example, at 40 ng of transfected expression plasmid, the AF2-containing chimera KER repressed transcription by 75% and ablation of AF2 by the L540Q mutation in the KERQ chimera resulted in 78% repression. Perhaps because of diminished ERE binding caused by the S554fs mutation (3), KERFS displayed reduced transcription repression, 69%, under the same conditions. The AF1 deletion modestly enhanced repression only when the KRAB domain was present at the C-terminus. At 40 ng of transfected expression plasmid transfected, the ERK and ΔA/B-ERK constructs repressed transfection by 78% and 88%, respectively. The KERK, KERQK and ΔA/B-ERK constructs were the most effective, with each repressing transcription 87-88%. Since these differences were negligible, we elected to use the KERK repressor in subsequent experiments.

KRAB Repression is not Blocked by Trichostatin A

It has been proposed that KRAB repression is mediated through recruitment of corepressors TIF1α and TIF1β, which appear to be involved in chromatin modification. Many chromatin modifiers recruit histone deacetylases (HDACs), or contain intrinsic HDAC activity. The HDAC inhibitor, Trichostatin A (TsA), has been widely used to identify chromatin events based on histone deacetylation (32,33). Addition of 0.25μM or 1μM TsA had no effect on the ability of KERK or GAL4K chimeras to repress transcription from several reporter genes (Fig. 3). While TsA failed to affect KRAB repression, several types of control experiments demonstrated that TsA was functional in these cells (data not shown). These data indicate that transcription repression by KRAB does not solely depend on histone deacetylase activity and other pathways may exist.

Effect of Cell Line, Promoter and Ligand on KRAB Repression

Thus far, we established that the KERK chimera was an effective repressor of transcription from an SV40-based promoter-enhancer in the ER negative HepG2 cell line. However, we wanted to determine further whether KRAB repression was equally effective on strong and weak promoters, whether the KERK chimera was an effective repressor in different cell backgrounds, and whether the KRAB chimera could repress transcription in the presence of wild-type ERα or ERβ (34,35). To examine the effect of promoter strength on KRAB repression, repression was evaluated on reporter genes containing the weak TK promoter, the strong SV40 promoter/enhancer and the extremely powerful EF1-α promoter. Repression in the presence of endogenous ER, was determined by cotransfecting expression plasmids into the cells along with the KRAB expression plasmid. Finally, transcription repression was tested in HepG2 cells (Fig. 4 panels A-C) and CHO cells (Fig. 4. panels C-F). Note that the ordinate of panel A is set on a logarithmic scale. As shown in

earlier studies (35,36), hERβ was significantly less effective in activating transcription than hERα (3.3 fold versus 15 fold), even though three times more hERβ expression plasmid than hERα expression plasmid was employed. hERα increased transcription 15, 2.6 and 0.9 fold on the $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40, and $(ERE)_4$-pGL3-EF1-α reporter plasmids respectively. This illustrates an inverse correlation between promoter strength and the additional contribution in promoter activity due to hERα activated transcription, until there was no additional ER-dependent transactivation with the powerful EF1-α promoter. However, in all instances increasing amounts of transfected KERK repressor fully repressed hERα or hERβ induced activity and most of the basal promoter activity. In the absence of hER, KERK repressed up to 82-92% of basal promoter activity on these reporter plasmids. When transfected at a 9 fold excess relative to hERα, KERK repressed TK promoter activity to 68% of the basal TK promoter activity, which is a 47 fold reduction from the hERα induced level of transcription.

Similar results were observed in the Chinese hamster ovary (CHO) cell line (Fig. 4D-F). In CHO cells, the TK, SV40 and *Xenopus* Vitellogenin B1 promoters were tested using the $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40PE, and pGL3-EREVIT reporter plasmids, respectively. These experiments suggested an interesting difference between transcription activation and repression. Even though the EREVIT promoter contained only one consensus ERE, two functional imperfect EREs and one non-functional imperfect ERE (37), while the other test promoters contained four consensus EREs, in CHO cells hERα activated transcription more potently from the EREVIT promoter, than from the other test promoters (3.4 fold versus 1.7-1.9 fold). These results may reflect the strong induction by

estrogen of the *Xenopus* Vitellogenin B1 promoter *in vivo* (38). However, transcription repression by the KRAB chimera was more closely correlated with the number of consensus EREs, and repression was somewhat more effective with the $(ERE)_4$-pGL3-TK and $(ERE)_4$-pGL3-SV40PE reporters than with the pGL3-EREVIT reporter. At a 1:1 ratio of transfected KERK and hERα, repression was clearly dominant as activity was reduced 3.3-3.6 fold relative to the activity in the presence of hERα alone (Fig. 4D-F).

A long-term goal of this work is the modification of estrogen receptor regulated gene expression in breast cancer cells. Therefore the ability of the KERK chimeras to repress transcription from the $(ERE)_4$-pGL3-TK and $(ERE)_4$-pGL3-SV40PE reporter genes in ER negative MDA-MB-231 human breast cancer cells was evaluated in the presence or absence of cotransfected hERα. Transfected KERK effectively suppressed the estrogen-induced activity and most of the basal activity of the reporter genes (Fig. 5A and B). Similar results were obtained in HeLa cells (data not shown). In MDA-MB-231 cells hERα induced transcription by 3.1 and 2.3 fold on the $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40PE reporters, respectively. KERK achieved maximal repression levels of 74-80% in the absence of hERα, and 61-75% in the presence of hERα.

To evaluate the ability of a KRAB chimera to repress transcription in cells containing high levels of endogenous ER, we tested the effectiveness of the KERK chimera in ER positive, MCF-7, human breast cancer cells (Fig. 6). The effectiveness of type I and type II antiestrogens in serving as KERK ligands to potentiate KRAB repression was also tested. Type I antiestrogens, such as 4-hydroxy tamoxifen (OHT) prevent the receptor LBD from

adopting a proper conformation required for interaction with AF2 dependent coactivators, but do not interfere with DNA binding. The type I antiestrogens exhibit partial agonist activity in some cell and promoter contexts. Type II or "pure" antiestrogens such as ICI 182,780 (ICI) or RU58,668 on the other hand, are thought to prevent nuclear translocation of hERα (39-41), which might seriously impair the ability of the KRAB repressor to repress transcription. To facilitate comparison of the ability of the different ligands to repress transcription, luciferase activity was set equal to 100% for each ligand in the absence of transfected KERK. Surprisingly, repression was not affected by the type of ligand used. Transcription was repressed most potently (8.8 fold) in the presence of ICI 182,780 compared to 4.8 and 4 fold in the presence of E_2 and TOT, respectively. Similar results were observed in the estrogen receptor negative HepG2 cell line (data not shown), suggesting that repression in MCF7 cells was truly due to active repression, and not just a result of interference of hER mediated transcription activation by antiestrogens.

Effect of Number of EREs and ERE Binding Affinity on Transcription Repression

Virtually all studies employing KRAB repressors have utilized conditions favorable to repression in which the KRAB chimera binds to synthetic constructs containing multiple copies of a perfect DNA binding site (8,10,12,13,24,25,27,42),. Since KERK repressed expression from the EREVIT promoter which contains a single consensus and three additional non-consensus EREs less effectively than it repressed promoters containing 4 consensus EREs (Fig. 4, D-F), it was of interest to establish the minimum number of consensus EREs required for repression. With this in mind SV40 based reporter genes containing 1, 2 and 4 EREs were constructed and the ability of transfected KERK to repress their transcription was examined. Repression was similar for the reporter genes

containing 2 or 4 EREs and reached a plateau at 87%. While repression from the reporter gene containing a single ERE was dose-dependent and effective, the inability to reduce promoter activity below ~30% of basal activity, even at high levels of transfected KERK was troubling (Fig. 7A). We therefore set out to enhance the potential of KERK to repress transcription.

Through the use of a modified form of the bacteriophage P22 challenge phage selection system (43), our laboratory identified progesterone receptor DNA binding domain mutations that changed the DNA binding specificity from the GRE/PRE to the ERE, and resulted in enhanced binding to the ERE (28). One of the PR DBD mutants isolated with enhanced affinity for the ERE was DBD5. DBD5 exhibits 15 fold higher affinity for the consensus ERE than the wild-type ER DBD. We reasoned that enhancing the ERE affinity of KERK would potentiate its transcription repression properties. Therefore the corresponding 3 mutations from the PR-DBD5 mutant were introduced into the DNA binding domain of KERK, resulting in KERK-3M. We compared the ability of KERK-3M and KERK to repress transcription from the promoter containing a single ERE. KERK-3M was a more potent repressor than KERK. Almost 2 fold less transfected KERK-3M was required to reach a given level of repression, and repression with KERK-3M did not plateau at the amounts tested. The KERK-3M chimera repressed transcription up to 4.8 fold, while KERK repressed transcription 2.7 fold under similar conditions (Fig. 7B).

KERK-3M, but not KERK, Effectively Represses Transcription from a Promoter Containing the Imperfect pS2 ERE

While these studies demonstrated that KERK and KERK-3M repressed transcription from a single consensus ERE, virtually all estrogen-regulated genes contain imperfect EREs. To evaluate whether KERK and KERK-3M could repress transcription from an imperfect ERE in a native promoter context, we elected to use the pS2 gene was used. Although pS2 is a clinical and prognostic marker for hormone responsive breast cancer (44), the function of pS2 and its role in breast cancer development and progression remain poorly understood. pS2 gene expression is strongly induced by estrogen and its promoter contains a single non-consensus ERE (5'-AGGTCActgTGGCCC-3') (26). Both *In vitro* binding and *in vivo* transactivation are substantially reduced when the nonconsensus pS2 ERE is present, rather than the consensus ERE (45). A 345 nucleotide fragment of the pS2 promoter containing the imperfect pS2 ERE was inserted upstream of the SV40 promoter in plasmid pGL3-Promoter (Promega). hER induced transcription of the reporter 2.8 fold the presence of 10 nM moxestrol. While, KERK could partially repress Mox-hER induced transcription, it was unable to suppress Mox-hER induced promoter activity back to the basal level (Fig. 8A). In striking contrast, the KERK-3M chimera however, effectively repressed all of the Mox-hER induced transcription and most of the basal promoter activity (Fig. 8B). These data indicate that use of a genetically selected set of mutations, which enhance binding to the ERE, strongly potentiates the ability of KRAB chimeras to repress transcription from the imperfect EREs found in native promoters.

DISCUSSION

KRAB Chimeras Containing ER Activation Domains Repress Transcription

Since the putative KRAB corepressor, TIF1α interacts with the AF2 domain of ligand occupied ER to act as an activator (21), KRAB repressor activity might be disrupted when coupled to wild-type ER. Precedent for this assumption was the observation that in a study of repression of the Human Immunodeficiency Virus-1 LTR, dominant negative Tat mutants linked to KRAB were far more effective repressors than Tat-KRAB chimeras retaining an active Tat transactivation domain (46). These data suggested that activation domains could interfere with the ability of the KRAB domain to repress transcription. We therefore constructed and tested a variety of ER-KRAB chimeras in which the KRAB domain was present at the N-terminus, the C-terminus, or at both ends of the protein, and AF1, AF2 or both activation domains were mutationally inactivated or deleted. All of the ER-KRAB chimeras effectively repressed transcription, indicating that the KRAB domain was dominant over the ER transactivation domains. It is not known whether the presence of the KRAB domain with its bound corepressors blocks coactivator binding to the ER, or whether the bound corepressors are dominant over ER coactivators. Since coactivators are known to interact with both AF1 and AF2 of ER (5,47), and the site of linkage of KRAB to the ER had little or no effect on KRAB repression, it is likely that the bound KRAB corepressors are dominant over any coactivators still able to bind the KRAB-ER chimeras.

KERK Represses Transcription when Wild-type ER is Present.

Even if the ER-KRAB chimeras and wild-type ER have similar affinities for the ERE, it seemed plausible that wild-type ER could compete effectively for binding to the EREs in our

reporter genes, and might block the ability of the KRAB chimeras to repress transcription. However, we found that KERK effectively represses transcription in the presence of either hERα or hERβ in several contexts. Even at a 1:1 ratio of transfected KERK to wild type hERα, most ER-induced transactivation is repressed (Fig. 4A-D). These data are consistent with our finding that the KRAB domain is dominant over the internal AF1 and AF2 domains of ER. It seems likely that in our reporters with multiple EREs, the presence of a mixed population of wild-type ER on some sites and KERK on other sites results in repression rather than activation.

Not only can KERK repress transcription in the presence of ER, it also represses transcription of a powerful ERE-containing promoter which is not estrogen inducible. In the progression of breast cancers to an estrogen-independent phenotype in which antiestrogens no longer limit their growth, it has been suggested that genes which were initially estrogen-regulated become constitutively active (48-50). The $(ERE)_4$-pGL3-EF1-α reporter serves as a prototype for this class of gene. Expression of the powerful EF1-α promoter is not up-regulated by estrogen receptor. However, KERK effectively suppresses the high level of basal transcription from this promoter (Fig. 4C). These data demonstrate that it is the presence of the ERE, rather than the capacity for estrogen induction which determines the potential for repression of a gene by a KRAB-ER chimera.

An ER Ligand is Required for Repression.

The role of ligand in ERE binding by ER has been controversial (reviewed in (51). While most studies support the view that liganded ER binds with higher affinity to the ERE than unliganded ER, using promoter interference assays some variable levels of ERE binding

by unliganded ER have been reported (51-53). Similar results were obtained with the KRAB repressor. A minimal level of repression was observed with unliganded KERK (Fig. 2B). However, the presence of either an estrogen agonist or antagonist strongly potentiated repression by KERK. The presence of the large KRAB repressor domain linked to the C-terminus of the ER does not appear to limit the access of ligand to the binding pocket. Our observation that KRAB-ER chimeras in which the KRAB was linked to either the N- or C-terminus had equal potency supports this view.

An important and unexpected finding was that both a type I mixed or partial agonist (OHT) and a type II "pure" antagonist (ICI 182,780) were as effective in inducing repression by the ER-KRAB chimeras as estrogens. Our data was surprising in light of observations that an important part of the action of type II antiestrogens stems from their ability to disrupt cytoplasmic-nuclear shuttling, causing hERα to be predominantly located in the cytoplasm (39,41,54). Further, this cytoplasmic receptor appears to be destroyed rapidly (39,54), essentially depleting cellular ER. This view is consistent with studies which use hER LBD in combination with Cre recombinase to achieve ligand activated site-specific recombination (55-57). In those studies specific LBD mutations (58) were required to activate recombination in the presence of type I or type II antiestrogens (56,57). We inserted these mutations into the KERK chimera and found no further enhanced repression in the presence of ICI or OHT (data not shown). Since KERK displayed a similar dose-dependent repression curve when liganded by E_2, OHT or ICI 182,780, our data suggest that similar amounts of repressor reach the nucleus in a functional form irrespective of the type of ligand used. It has been shown that ICI 182,780 occupied receptor can bind DNA *in vitro*, albeit with slowed kinetics (59), and another *in vivo* study

suggests that at least part of the receptor population occupied with type II antiestrogens is still able to bind the ERE (52). One explanation for the potent repression we see in the presence of ICI 182,780 is that nuclear localization is potentiated by the KRAB corepressor TIF1α. In a study using an ER mutant missing the nuclear localization signal, the TIF1α coactivator allowed ligand-dependent nuclear localization (21). Since TIF1α is a putative KRAB corepressor it may carry out a similar function and nuclear localize ICI 182,780 occupied ER-KRAB chimeras.

The Histone Deacetylase Inhibitor, Trichostatin A, Does not Interfere with Repression by the KRAB Domain

One possible explanation for the ability of the KRAB domain to repress transcription is that it recruits a corepressor complex containing histone deacetylase activity. It is widely accepted that deacetylated histones interact more tightly with DNA and induce a repressive chromatin formation. Since the histone deacetylase inhibitor, trichostatin A (32,33) had not been used in conjunction with KRAB repression previously, we examined its ability to interfere with repression by the KRAB domain. The inability of two concentrations of TsA to affect repression by two KRAB chimeras on several promoters indicates that KRAB action is not limited to the recruitment of HDACs. Recently, Xue and colleagues isolated an ATP dependent chromatin remodeling complex containing histone deacetylase activity called NURD (60). Interestingly, a fusion of the GAL4 binding domain with one of the proteins identified in the complex, MTA1, repressed transcription. This repression could not be relieved by TsA, which suggests that pathways of repression exist that do not depend on HDAC activity. Temporal aspects of the repression process may also underlie the failure of TsA to relieve KRAB mediated repression. While HDACs may be involved during the

adoption of the repressed chromatin structure, they may not be critical for its maintenance. In this regard, histone acetylases involved in the relief of repression may be prevented access to their substrate by heterochromatin-enriched factors HP1a, MOD1 and MOD2 that reportedly interact with KRAB corepressors TIF1α and TIF1β (17,61,62).

Binding to a Single ERE is Sufficient for KRAB Repression

Our studies show that a Gal 4-KRAB chimera and an ER-KRAB chimera each exhibit sequence-specific repression and that changing the DNA binding specificity of an ER-KRAB chimera abolishes KRAB repression. Therefore, it is clear that ERE binding is required for KRAB repression. Evidence that KRAB repression is sensitive to ERE binding affinity came from our finding that KERFS was a less effective repressors than the similar KERQ chimera. While both the S554fs and L540Q mutations disrupt the AF2 transactivation domain, previous studies have shown that S554fs has a reduced affinity for the ERE (3). The low concentration of the KERK chimera required for repression suggests that the presence of the KRAB domain did not significantly reduce affinity of the ER for the ERE.

Previous studies with KRAB chimeras used promoters with multiple (four to seven) binding sites, raising the possibility that a large number of KRAB repressor domains need to be bound to the DNA for efficient repression of transcription (13,25). This is the first study to demonstrate that a single DNA binding site is sufficient for KRAB mediated repression. Repression from a single response element is critical for potential future applications of ER-KRAB chimeras to studies of estrogen-regulated genes. Our studies suggest that tight binding to a DNA recognition sequence may be more critical for transcription repression than for transcription activation. It appears that for transcription activation, synergism can mask diminished binding (63), which is not the case for KRAB-

mediated repression. Two EREs were clearly more effective in enabling repression by KERK than a single ERE, but there was no further increase in repression in going from 2 to 4 EREs. This is in stark contrast to hER-mediated transcription activation, where strong synergistic effects were observed comparing activity on reporter genes containing 1, 2 and 4 EREs (63,64). Additional support for the idea that tight binding to a response element is important for repression comes from studies with the promoter fragment containing the pS2 ERE. Several studies have shown that ER binds to the pS2 ERE with a lower affinity than to the consensus ERE (26,45). Despite this diminished binding, hER achieved a 2.8 fold transcription activation on the pS2 ERE. In striking contrast KERK was completely unable to suppress basal promoter activity when bound to the same pS2 ERE. The limited ability of KERK to suppress ER-mediated induction of the reporter containing the pS2 ERE, may stem from the ability of KERK to act as a dominant negative mutant interfering with binding of the wild type ER, without exerting active repression. KERK-3M however, achieved effective transcription repression on the pS2 ERE, suggesting that high affinity binding to the imperfect ERE, resulting in a continued presence the ER-KRAB chimera on the promoter is critical for repression. These results can be explained in one of several ways. In transcription activation, the recruitment of HAT complexes to the DNA by transcription factors stabilizes the ternary complex. Promotion of initial transcription factor binding is thought to involve a low level of sporadic nucleosomal acetylation, which nucleates the efficient binding of transcription factors upon transcriptional induction (65). This activity is especially important for genes driven by weak binding sites and may explain transcriptional synergism observed with multiple weak binding sites. We expect to see the reverse should repression by KRAB mainly occur via chromatin condensation. In this case, the increased ERE binding affinity may allow KERK-3M to bind DNA better than KERK in the

context of a condensed chromatin structure, thereby ensuring that the condensed structure, once adopted is maintained. The ability of the KRAB domain to repress transcription from a single binding site and when bound far from the site of transcription initiation may be explained by the recent discovery of loop domains formed by the SWI/SNF chromatin remodeling complex. This suggests that upon formation of the loop, nucleosomes may be sequentially disrupted by SWI/SNF through linear diffusion. Alternatively, an increased interaction of KRAB corepressors with factors in the transcription initiation complex due to prolonged presence on the promoter of KERK-3M could explain our observations. Our finding that different rules appear to apply for transcription activation and repression indicate that further study into the mechanisms by which KRAB represses transcription and the role of DNA binding affinity in that process are warranted.

Our work demonstrates a novel approach to ligand-dependent repression of target genes. We have shown that ER chimeras containing the Kox 1 KRAB repressor domain efficiently repressed transcription of ERE-containing promoters. Since binding can be potentiated by estrogens and by widely used antiestrogens, ligand-dependent modulation of gene activity is feasible. However, chimeras containing the wild-type ER DBD were unable to repress transcription of a reporter gene containing the fragment from the pS2 5'-flanking region which contains the imperfect pS2 ERE. It was only by combining previously by genetic selection identified DBD mutations that confer enhanced ERE binding properties with the KRAB repressor system that we were able to create a novel ligand-dependent repression system for modulating the activity of ER-regulated genes. The unique characteristics of the hER-KRAB chimeras we describe make them powerful new tools for the functional analysis of ER-regulated genes.

MATERIAL AND METHODS

Cloning of hERα-KRAB chimeras

To fuse the Kox1 KRAB domain to hERα, unique NheI sites were introduced into the hERα cDNA sequence. To facilitate sequence verification after mutagenesis, the following hER fragments from plasmid pCMV5hER were initially subcloned into pGEM11Zf(+) (Promega): 1) the N-terminal fragment EcoRI/NotI; 2) the NotI/HindIII fragment containing the LBD; 3) the HindIII/BamHI C-terminal fragment of pCMV5hER and pCMV5hERL540Q (3,66). Quikchange mutagenesis (Stratagene) then was employed to generate unique NheI sites in these plasmids, generating the vectors pG11EnsNhe, pG11EnhNhe, pG11EbhNhe and pG11QbhNhe, respectively. To achieve this the following primers were used:

For pG11EnsNhe:

Forward: GCCCGCGGCCACGGACCGCTAGCAATGACCATGACCCTCCA

Reverse: TGGAGGGTCATGGTCATTGCTAGCGGTCCGTGGCCGCGGGC

For pG11EnhNhe

Forward: AAGTATGGCTATGGAGCTAGCCAAGGAGACTCGCTA

Reverse: TAGCGAGTCTCCTTGGCTAGCTCCATAGCCATACTT

For pG11EbhNhe and pG11QbhNhe.

Forward: GAGGCAGAGGGTTTCCTGCTAGCTGCCACAGTCTGAG

Reverse: CTCAGACTGTGGCAGCTAGCAGGAAACCCTCTGCCTC

The Kox1 cDNA (9) was a kind gift of Dr. Hans-Jürgen Thiesen (University of Rostock, Germany). PCR amplification by Taq DNA polymerase (Life Technology) generated fragments of the Kox1 (ZNF10) protein (aa 1-91) containing both the KRAB A and

KRAB B domains, that could be cloned at either the N-terminus of hER and ΔA/B-hER (N-KRAB), or at the C-terminus of hER and hERL540Q (C-KRAB). The following oligonucleotides were used:

N-KRAB

Forward: CAGAATTCATGGATGCTAAGTCACTAAC,

Reverse: TATCTAGAAATGCAGTCTCTGAATCAG

C-KRAB

Forward: CTTCTAGATATGGATGCTAAGTCACTAAC

Reverse: ATGGATCCTAAATGCAGTCTCTGAATCAG

The resulting amplified products were subcloned into the pGEM T-vector (Promega). After verifying the sequence, the N-KRAB insert was obtained as an EcoRI/XbaI fragment and together with either the NheI/NotI fragment of plasmid pG11EnsNhe cloned into pCMV5hER, pCMV5hERL540Q and pCMV5hERfs digested with EcoRI/NotI or with the NheI/HindIII fragment of pG11EnhNhe into pCMV5hER, pCMV5hERL540Q and pCMV5hERfs digested with EcoRI/HindIII. These manipulations yielded plasmids pCMV5KER, pCMV5KERQ, pCMV5KERFS, pCMV5K-ΔA/B-ER, pCMV5K-ΔA/B-ERQ, and pCMV5K-ΔA/B-ERFS, respectively. The C-KRAB insert was obtained as an XbaI/BamHI fragment and ligated into NheI/BamHI digested plasmids pG11EbhNhe and pG11QbhNhe, respectively. The resulting hER LBD-KRAB fusions then were obtained as XbaI/BamHI fragments and cloned into similarly digested plasmids pCMV5hER, pCMV5ΔA/B-hER, pCMV5KER and pCMV5K-ΔA/B-ER. These manipulations yielded plasmids pCMV5ERK,

pCMV5ERQK, pCMV5ΔA/B-ERK, pCMV5ΔA/B-ERQK, pCMV5KERK, pCMV5KERQK, pCMV5K-ΔA/B-ERK and pCMV5K-ΔA/B-ERQK, respectively.

To establish that ERE binding is required for transcription repression by the ERK chimera, its wild-type hER DNA binding domain was replaced through exchange of the respective NotI/HindIII fragments with a mutated DNA binding domain. This latter DBD no longer recognizes the ERE sequence due to the E203G, G204S and A207V mutations in the DNA recognition helix (6,29). To establish that a functional KRAB domain is required for transcription repression, the previously reported E26A, E27A and E28A mutations (8) were introduced into the KRAB domain of the ERK chimera with the Quikchange protocol using the following oligonucleotides:

Forward: GACTTCACCAGGGCGGCCGCGAAGCTGCTGGAC

Reverse: GTCCAGCAGCTTCGCGGCCGCCCTGGTGAAGTC

A Flag-GAL4-KRAB chimera was constructed to serve as a control. Dr. C.M. Chiang (University of Illinois) provided us with a Flag-GAL4-VP16 fusion construct in the bacterial expression plasmid pET11d (Novagen). We obtained the Flag-GAL4-VP16 coding sequence, by digestion with NcoI and subsequent fill in with PfuI polymerase, followed by BamHI digestion to liberate the insert. The gel purified fragment then was ligated into the mammalian expression vector pCDNA3 (Stratagene) to generate plasmid pFGVP16. For this purpose, pCDNA3 was initially digested with HindIII, filled in with Pfu polymerase and subsequently digested with BamHI. The GAL4 C-terminus was obtained in conjunction with a polylinker as a PCR fragment from the plasmid pM (Clontech), changing the Dam methylation sensitive BclI site into an ApaI site in the process. The PCR fragment was digested with XhoI/ApaI and ligated into the similarly digested plasmid pFGVP16 to

generate plasmid pFGmcs. In our transfections, this plasmid is referred to as GAL4. The above described N-terminal KRAB domain, obtained as an EcoRI/BamHI fragment, was ligated into plasmid pFGmcs, which provided the stop codon, generating the vector pFGK.

The plasmid $(ERE)_4$-pGL3-SV40PE constructed served as an indicator of repression. This plasmid derived from plasmid pGL3-Control (Promega, Madison, WI), contains four consensus EREs upstream of the SV40 promoter, which rendered the plasmid estrogen responsive. Together, the SV40 promoter and enhancer in this plasmid, constitutively drive the expression of firefly luciferase, therefore both activation and repression can be studied effectively. The estrogen response elements were obtained from plasmid $(ERE)_4$-TATA-CAT (64), which was digested with HindIII, blunt ended with Pfu polymerase and religated to generate an NheI site. Subsequently, an NheI/BglII digest was performed to liberate the EREs. This fragment then was ligated into the similarly digested vector pGL3-Control (Promega). Another series of pGL3-Control based reporters was constructed containing one, two and four ERE copies, respectively. To achieve this, an extraneous BglII site was removed from the multiple cloning site of plasmids pGL3-$(ERE)_1$-TATA, pGL3-$(ERE)_2$-TATA and pGL3-$(ERE)_4$-TATA (28) by HindIII/XhoI digestion and subsequent religation after Pfu DNA polymerase mediated fill in. Following this treatment, the BglII/SalI backbone fragment containing the respective number of EREs was ligated to the BglII/SalI fragment of the BglII/PvuI/SalI digested plasmid pGL3-Control. To test the ER-KRAB chimeras in a non-SV40 based promoter/enhancer context, the plasmids pGL3-E_4-TK and pGL3-E_4-EF1-α were constructed. Plasmid pGL3-TK was constructed by inserting the TK promoter/enhancer as a BglII/HindIII fragment obtained from the plasmid PRL-TK (Promega) into similarly digested plasmid pGL3-Basic (Promega). Plasmid pGL3-EF1-α was constructed by inserting the EF1-α promoter/enhancer obtained as a HindIII/NcoI

fragment from the plasmid pEFmyc/nuc (Invitrogen) into similarly digested plasmid pGL3-Basic. These plasmids then were made estrogen responsive by incorporating four copies of the ERE obtained as an NheI/BglII fragment from the plasmid pGL3-$(ERE)_4$-TATA. For studies with the Flag-GAL4-KRAB fusions, the repression reporter plasmid G_5-pGL3-Control was constructed by inserting five GAL4 binding sites obtained as an XhoI/BamHI fragment from the plasmid pG5E1b (67), into XhoI/BglII digested plasmid pGL3-Control.

Cell maintenance, transfection and reporter gene assays

HepG2, human hepatoma cells and HeLa cells were maintained in a humidified 5% CO_2 containing environment at 37°C in Dulbecco's Minimal Essential Medium (Sigma) supplemented with 10% charcoal dextran stripped fetal bovine serum (Atlanta Biologicals) 50,000 U/l penicillin and 50 mg/l streptomycin (Gibco/BRL). CHO (Chinese Hamster Ovary) cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, 1:1 (Sigma), 29.2 mg/l L-Glutamine (Sigma), 5% charcoal dextran stripped newborn bovine serum (Atlanta Biologicals), 50,000 IU/l penicillin and 50 mg/l streptomycin. MDA-Mb-231, human breast cancer cells were maintained in Leibovitz's L-15 medium with 10 mM HEPES, 5% Newborn calf serum, 50,000 U/l penicillin, 50 mg/l streptomycin (Gibco/BRL), 25 mg/l Gentamycin, 6 µg/l insulin, 3.75 µg/l hydrocortisone and 16 mg/l glutathione. At least two days prior to the experiment, the cells were transferred to Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, 1:1 (Sigma), 29.2 mg/l L-Glutamine (Sigma), 5% charcoal dextran stripped newborn bovine serum (Atlanta Biologicals), 50,000 U/l penicillin 50 mg/l, streptomycin (Gibco/BRL), 25 mg/l Gentamycin, 6 µg/l insulin, 3.75 µg/l hydrocortisone and 16 mg/l glutathione. MCF-7 cells were maintained in Eagle's minimum essential medium plus phenol red, supplemented with 5% newborn calf serum, 50,000

IU/l penicillin and 50 mg/l streptomycin. At least two days prior to the experiment, cells were transferred to Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12, 1:1 (Sigma), 29.2 mg/l L-Glutamine (Sigma), 5% charcoal dextran stripped newborn bovine serum (Atlanta Biologicals), 50,000 IU/l penicillin and 50 mg/l streptomycin.

Transient transfections were carried out by the calcium phosphate co-precipitation method. Briefly, cells were plated in either 60 mm dishes, at a density of 4.5×10^5 cells/dish for HepG2 cells and 2.5×10^5 cells/dish for CHO cells, in 6-well plates at 1.0×10^5 cells/well, or in 12-well plates at 5×10^4 cells/well, respectively. The next day, the media was replaced and 2-6 h later the calcium phosphate crystals were added. 12-16 hours later, the cells were subjected to a three minute shock with 20% glycerol in a Tris buffered saline solution, pH 7.4 (TBS). Medium was replaced and where appropriate, hormone was added to the indicated concentrations. The cells were harvested 48 h later for the reporter gene assay by addition of appropriate amounts of passive lysis buffer (Promega). The activity of the resulting extracts was determined using the dual luciferase assay protocol (Promega) according to the manufacturers directions on a Monolight 2010 luminometer.

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FIGURE LEGENDS

Fig. 1. Repression by ERK is sequence-specific, requires ligand and a functional KRAB domain.

(A) Transcription repression properties of the KERK chimera and activation properties of hERα on the $(ERE)_4$-pGL3-SV40PE reporter plasmid in HepG2 cells in the absence and presence of the agonist Moxestrol (10 nM). All other experiments were carried out in the presence of 10 nM Mox, except where noted. Luciferase activity from the transfected reporter was determined as described in Methods and the reporter plasmid alone was set at 100%. To establish whether both sequence-specific DNA binding and a functional KRAB domain are required for repression by the ERK chimera, the effects on transcription from the $(G)_5$-pGL3-SV40PE and the $(ERE)_4$-pGL3-SV40PE reporter plasmids in HepG2 cells were examined by cotransfection of the indicated GAL4 DBD and hER based effector constructs (Panel B and C, respectively). The data obtained were normalized against the luciferase activity of the indicated reporter plasmid alone which was set equal to 100%. The data in panels A-C represents the average of at least three independent transfections $\pm$ s.e.m.

Fig. 2. Influence of AF1 and AF2 on Repression Properties of hER-KRAB Chimeras.

(A) The KRAB domain was fused in frame at either the N- or C-terminus and at both termini of the full-length wild-type hERα, at the N-terminus of several hERα mutants in which the ligand independent activation function (AF1) was removed through deletion of the A/B domain (ΔA/B), or in which the ligand dependent activation function (AF2) was ablated by point mutations L540Q (Q) or S554fs (FS), or a combination of these two classes of mutations. In the constructs, the DNA binding domain (DBD) is indicated as a cross-

hatched box, the AF2 mutations in the ligand binding domain (LBD) are indicated as Q (L540Q) and FS (S554fs) respectively. Ablation of AF1 activity, achieved through deletion of the first 178 amino acids of hERα is indicated as ΔA/B. The KRAB repressor domain is indicated as a black box. (B) Increasing amounts (5, 20 or 40 ng) of the expression plasmids encoding the hER-KRAB chimeras were transfected into HepG2 cells using the $(ERE)_4$-pGL3-SV40PE plasmid as a reporter. A vertical line in the ER LBD indicates the L540Q point mutation (Q), while the horizontally hatched box extending the LBD C-terminus, represents the additional amino acid sequence introduced by the S554fs point mutation (FS). The data obtained was normalized against the luciferase activity of the reporter plasmid alone, which was arbitrarily set at 100%. The data in panel B represents the average of at least three independent transfections ± s.e.m.

Fig. 3. Trichostatin A does not interfere with repression by KRAB chimeras

To establish whether Trichostatin A could relieve KRAB mediated repression, the effects in HepG2 cells on transcription from cotransfected reporter plasmids $(G)_5$-pGL3-SV40PE and $(ERE)_4$-pGL3-SV40PE and the indicated cotransfected GAL4 DBD and hER based effector constructs in the absence (open bars) or presence (0.25 μM, hatched bars; 1 μM, filled bars) of Trichostatin A were examined. Trichostatin was added 24 h prior to harvest where appropriate. The data represent the average of at least 3 independent transfections ± s.e.m.

Fig. 4. KERK effectively represses transcription on several estrogen responsive promoters in HepG2 and CHO cells in the presence and absence of hER.

Repression was assessed in the presence of 10 nM Moxestrol in the presence and absence of the indicated amounts of cotransfected hERα or hERβ expression plasmids using the reporter plasmids $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40PE, and $(ERE)_4$-pGL3-EF1-α in HepG2 cells (panels A-C, respectively), and plasmids $(ERE)_4$-pGL3-TK, $(ERE)_4$-pGL3-SV40PE, and pGL3-EREVIT in CHO cells (panels, D-F, respectively). The transfections and luciferase assays were carried out as described in Materials and Methods. The data represents the average of at least three independent transfections ± s.e.m., normalized to the activity of the indicated reporter plasmid alone, which was set equal to 100%.

Fig. 5. Transcription repression by the KERK chimera in the estrogen receptor negative breast cancer cell line, MDA-MBR 231.

Repression was assessed in the presence of 10 nM Moxestrol, in the presence and absence of indicated amounts of cotransfected hERα using the reporter plasmids $(ERE)_4$-pGL3-TK and $(ERE)_4$-pGL3-SV40PE (panels A and B, respectively). The transfections and luciferase assays were carried out as described in Materials and Methods. Luciferase activity of the indicated reporter plasmid alone, was set equal to 100%. The data represents the average of at least three independent transfections ± s.e.m.

Fig. 6. Antiestrogens induce repression by KERK in MCF-7, human breast cancer cells. Repression was assessed on the $(ERE)_4$-pGL3-SV40PE reporter plasmid in the presence of either 17b-estradiol (10 nM), OHT (10 nM) or ICI 182,780(10 nM), respectively. Transfections and luciferase assays were carried out as described in Materials and Methods. To facilitate comparisons, the data obtained for each individual treatment group

was normalized against the luciferase activity of the reporter plasmid alone in the absence of transfected chimera and was set at 100%. The data represent the average ± s.e.m. for at least 3 independent transfections.

Fig. 7. Repression from a single consensus ERE is increased when ERE binding of the chimera is enhanced. Repression was assessed in the presence of 10 nM E_2. Transfections and luciferase assays were carried out as described in Materials and Methods. In all cases, the data obtained was normalized against the luciferase activity of the indicated reporter plasmid alone, which was set at 100%. In panel A, transcription repression by the KERK chimera was assessed in HepG2 cells on pGL3-SV40PE reporter plasmids containing the indicated number of EREs. Panel B shows repression in HepG2 cells by KERK and by a mutant KERK possessing increased DNA binding, KERK-3M, on the $(ERE)_1$-pGL3-SV40PE plasmid cells. Note that the data for the KERK chimera is also shown in panel A.

Fig. 8. Transcription repression from a promoter containing the pS2 ERE. The pGL3-pS2/SV40P reporter gene was transfected into HepG2 cells in the presence or absence of co-transfected hER and 10 nM Mox. The activity of the reporter gene in the absence of any transfected repressor or hER was set at 100%. Panel A shows repression by the indicated amounts of transfected KERK expression plasmid and panel B shows repression by the KERK-3M plasmid. The data represents the average ± s.e.m. for at least 3 independent transfections.

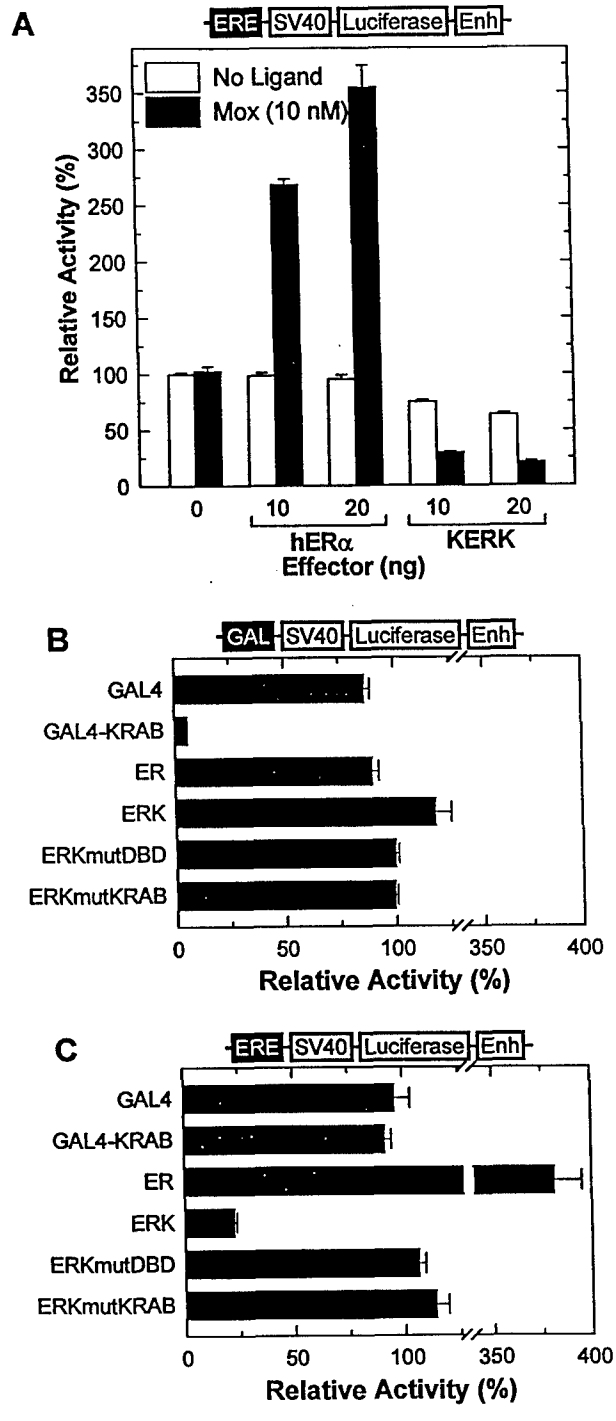
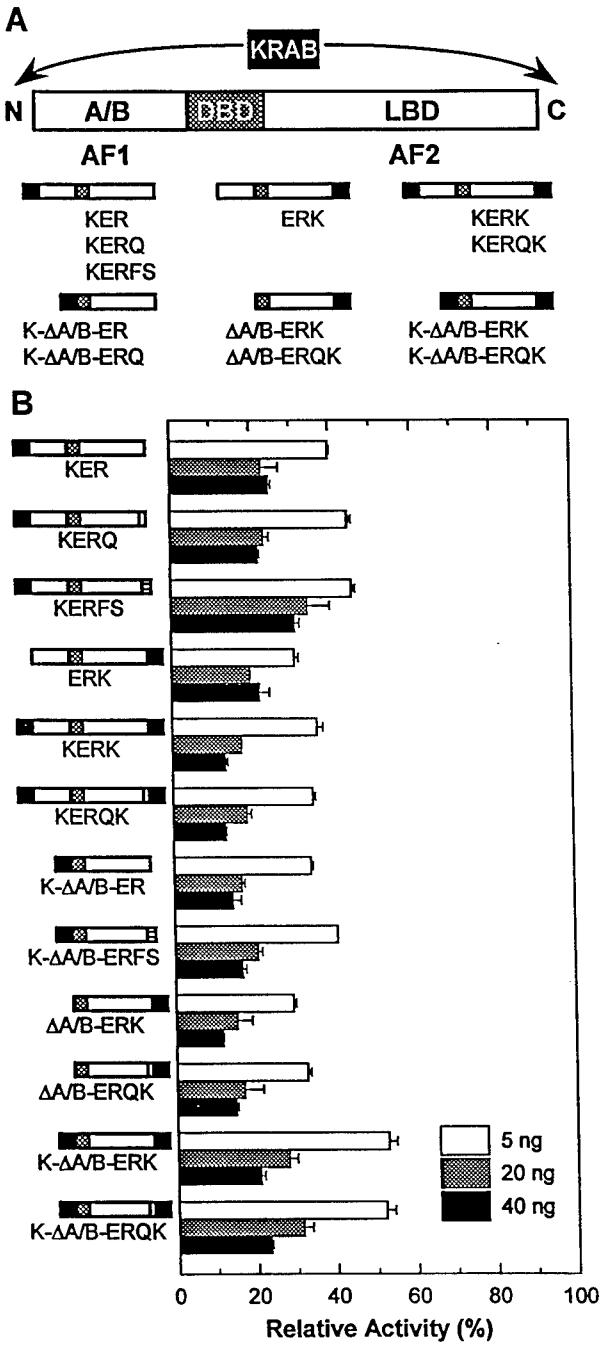

A
ERE
SV40
Luciferase
Enh
No Ligand
Mox (10 nM)
Relative Activity (%)
350
300
250
200
150
100
50
0
0
10
20
10
20
hERα
KERK
Effector (ng)
B
GAL
SV40
Luciferase
Enh
GAL4
GAL4-KRAB
ER
ERK
ERKmutDBD
ERKmutKRAB
0
50
100
350
400
Relative Activity (%)
C
ERE
SV40
Luciferase
Enh
GAL4
GAL4-KRAB
ER
ERK
ERKmutDBD
ERKmutKRAB
0
50
100
350
400
Relative Activity (%)

Figure 1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KRAB
N
A/B
DBD
LBD
C
AF1
AF2
KER
KERQ
KERFS
ERK
KERK
KERQK
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K-ΔA/B-ERQ
ΔA/B-ERK
ΔA/B-ERQK
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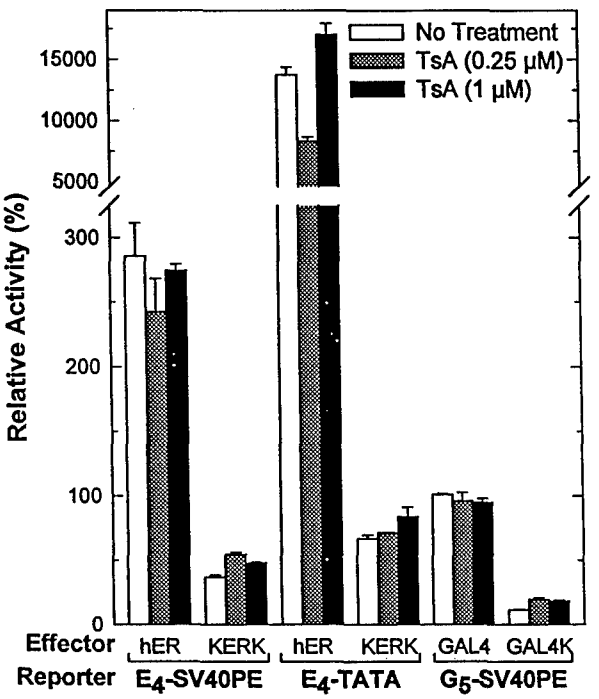
B
KER
KERQ
KERFS
ERK
KERK
KERQK
K-ΔA/B-ER
K-ΔA/B-ERFS
ΔA/B-ERK
ΔA/B-ERQK
K-ΔA/B-ERK
K-ΔA/B-ERQK
5 ng
20 ng
40 ng
0
20
40
60
80
100
Relative Activity (%)

Figure 2


No Treatment
TsA (0.25 μM)
TsA (1 μM)
Relative Activity (%)
15000
10000
5000
300
200
100
0
Effector
Reporter
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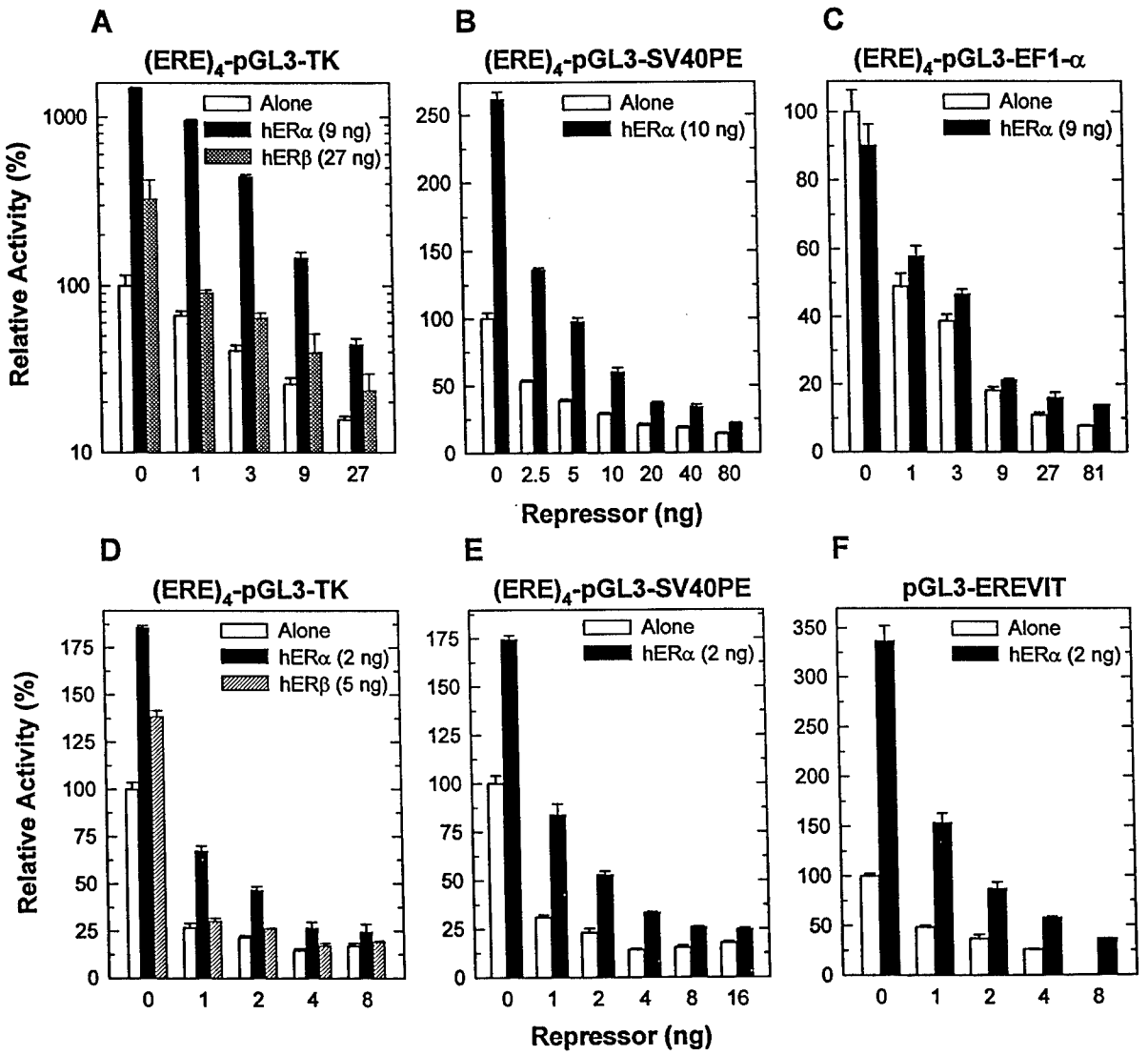
KERK
hER
KERK
GAL4
GAL4K
E4-SV40PE
E4-TATA
G5-SV40PE

Figure 3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Alone
hERα (9 ng)
hERβ (27 ng)
Relative Activity (%)
1000
100
10
0 1 3 9 27
B
(ERE)4-pGL3-SV40PE
Alone
hERα (10 ng)
250
200
150
100
50
0
0 2.5 5 10 20 40 80
C
(ERE)4-pGL3-EF1-α
Alone
hERα (9 ng)
100
80
60
40
20
0
0 1 3 9 27 81
Repressor (ng)
D
(ERE)4-pGL3-TK
Alone
hERα (2 ng)
hERβ (5 ng)
Relative Activity (%)
175
150
125
100
75
50
25
0
0 1 2 4 8
E
(ERE)4-pGL3-SV40PE
Alone
hERα (2 ng)
175
150
125
100
75
50
25
0
0 1 2 4 8 16
F
pGL3-EREVIT
Alone
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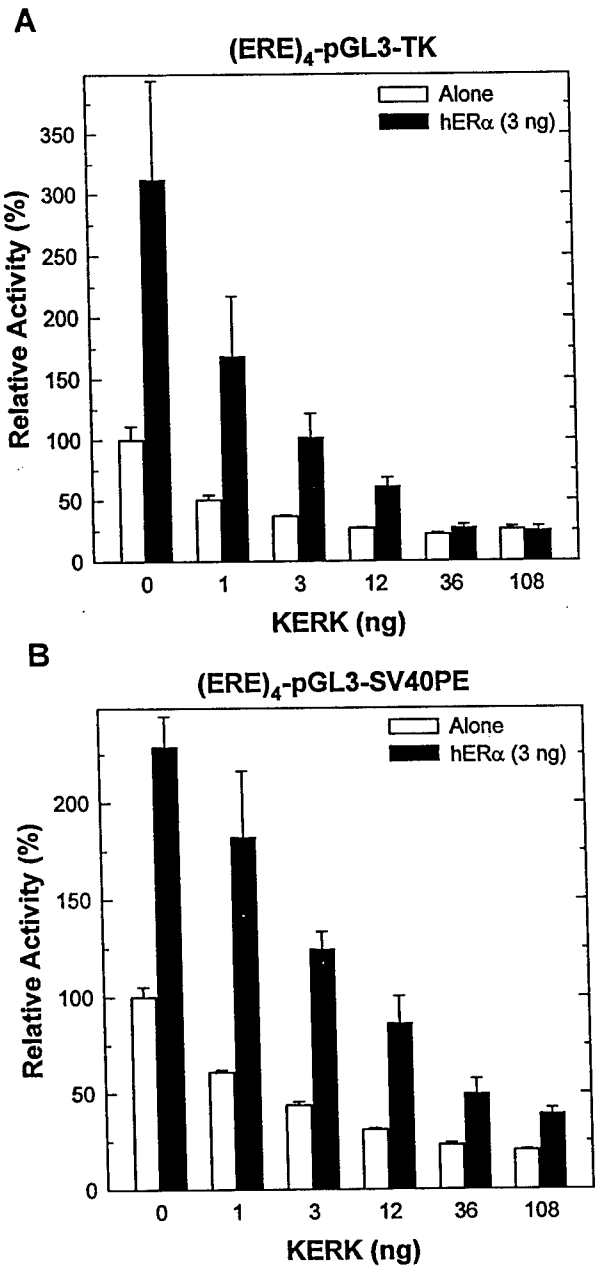
350
300
250
200
150
100
50
0
0 1 2 4 8
Repressor (ng)

Figure 4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(ERE)4-pGL3-TK
Alone
hERα (3 ng)
Relative Activity (%)
350
300
250
200
150
100
50
0
0
1
3
12
36
108
KERK (ng)
B
(ERE)4-pGL3-SV40PE
Alone
hERα (3 ng)
Relative Activity (%)
200
150
100
50
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0
1
3
12
36
108
KERK (ng)

Figure 5

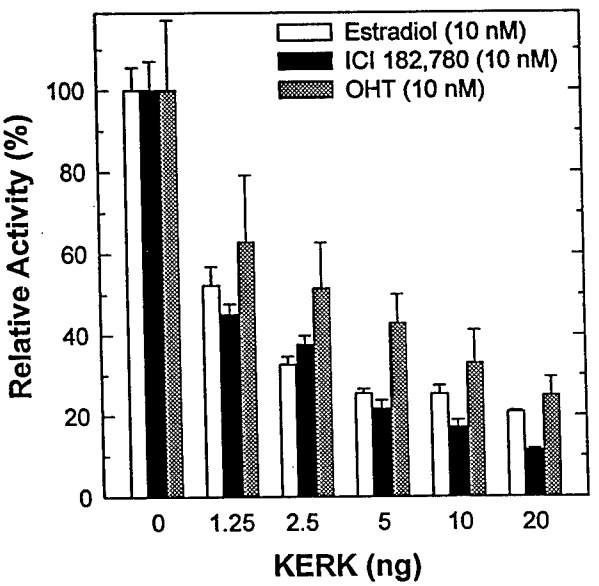
Estradiol (10 nM)
ICI 182,780 (10 nM)
OHT (10 nM)
Relative Activity (%)
100
80
60
40
20
0
0
1.25
2.5
5
10
20
KERK (ng)

Figure 6

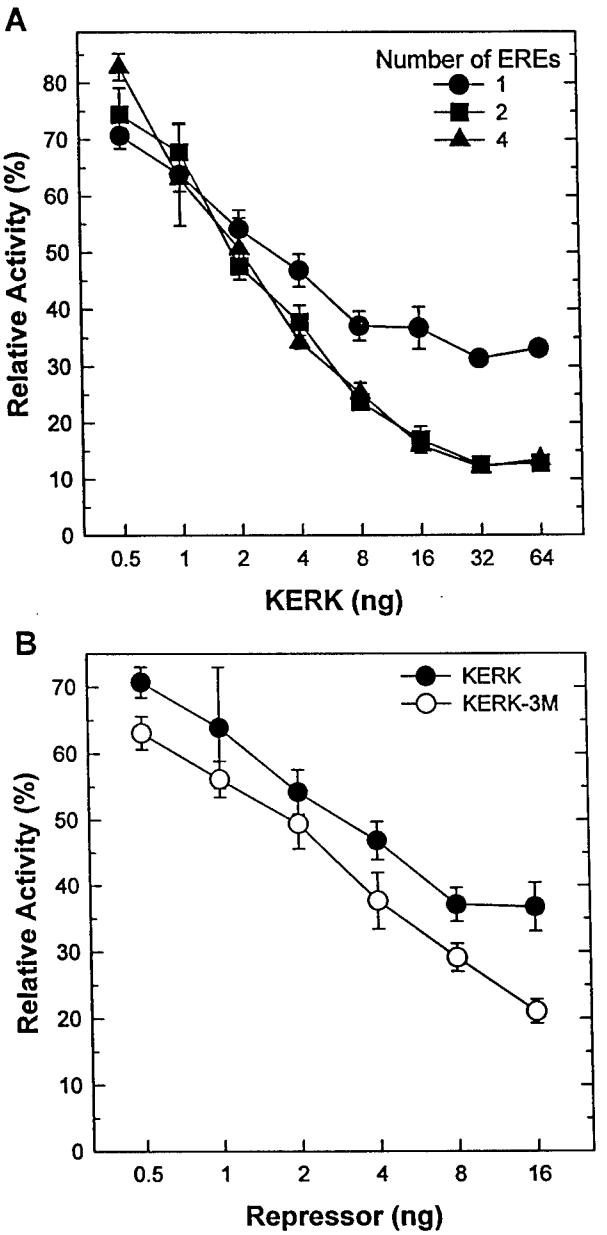
A
Number of EREs
1
2
4
Relative Activity (%)
0
10
20
30
40
50
60
70
80
0.5
1
2
4
8
16
32
64
KERK (ng)
B
KERK
KERK-3M
Relative Activity (%)
0
10
20
30
40
50
60
70
0.5
1
2
4
8
16
Repressor (ng)

Figure 7

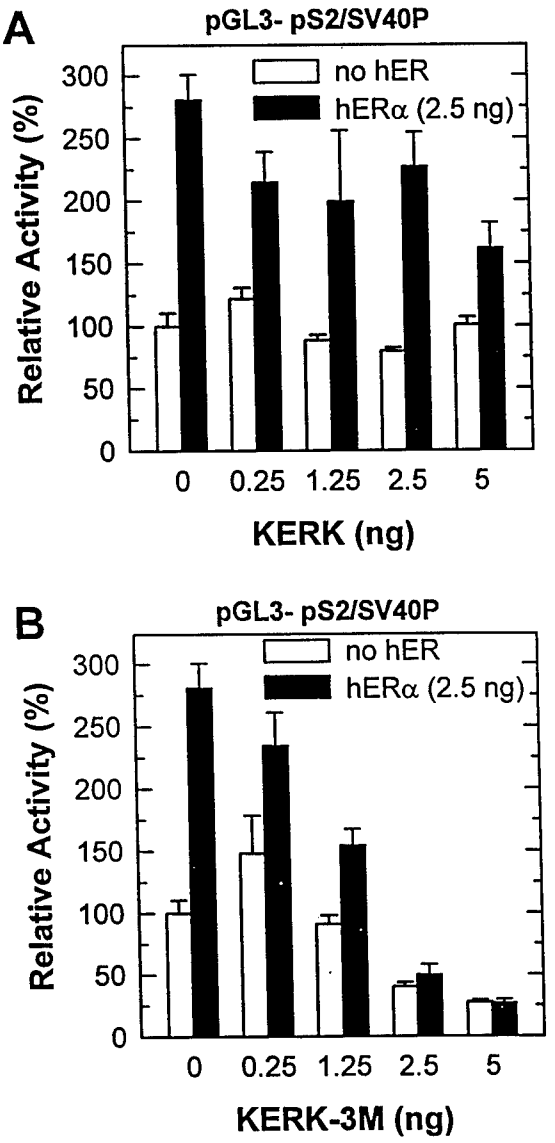
A
pGL3- pS2/SV40P
no hER
hERα (2.5 ng)
Relative Activity (%)
300
250
200
150
100
50
0
0
0.25
1.25
2.5
5
KERK (ng)
B
pGL3- pS2/SV40P
no hER
hERα (2.5 ng)
Relative Activity (%)
300
250
200
150
100
50
0
0
0.25
1.25
2.5
5
KERK-3M (ng)

Figure 8

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 23 Aug 01

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the technical reports listed at enclosure. Request the limited distribution statement for these reports be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Reports to be Downgraded to Unlimited Distribution

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